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Paralytic shellfish poison accumulation yields and feeding time activity in the Pacific oyster (*Crassostrea gigas*) and king scallop (*Pecten maximus*)

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Abstract

Pacific oysters and king scallops placed individually in a recirculating flume were fed for 2 weeks with a constant concentration (120 cell ml^{-1}) of a toxic strain of *Alexandrium minutum*. Fluorescence at the outlet of each experimental unit was measured continuously, and biodeposits were recovered twice daily to evaluate feeding time activity (FTA) and rates of organic filtration (OFR), ingestion (OIR) and organic absorption (OAR). Ion-pairing high performance liquid chromatography (IP-HPLC) was performed concurrently to quantify paralytic phycotoxins both (i) individually in shellfish at the end of the contamination period and (ii) in *A. minutum* cultures to estimate cellular toxin concentration. These data allowed comparison of the actual tissue toxin concentration (TOX) with the theoretical toxin accumulation rate (TAR) calculated from the linear relations between OAR, cell number, fluorescence and cell toxicity. The results show high FTA/TAR and FTA/TOX correlations ($R^2 = 0.78$) for both oysters and scallops. The TAR/TOX relation, though not yet clearly defined, suggests the minimum quantity of absorbed toxin at which the accumulation process is induced.

Résumé

Rendements d'accumulation des toxines paralysantes (IPFM) et fréquence d'activité nutritionnelle de l'huître creuse (*Crassostrea gigas*) et de la coquille Saint-Jacques (*Pecten maximus*). Des huîtres creuses et des coquilles Saint-Jacques ont été placées individuellement dans un circuit fermé et nourries pendant 2 semaines avec un apport constant d'*Alexandrium minutum* (souche toxique) à la concentration de 120 cell ml^{-1}. La fluorescence en sortie de chaque enceinte expérimentale a été mesurée en continu et la production de biodépôts évaluée 2 fois/j afin de calculer la fréquence d'activité alimentaire (FTA), le taux de filtration organique (OFR), le taux d'ingestion et d'absorption organique (OIR et OAR). Des analyses en chromatographie liquide par appariement d'ions ont été réalisées en même temps afin de quantifier les concentrations de toxines paralysantes présentes : dans les coquillages contaminés en fin de période de contamination et dans les cultures d'*A. minutum*. Ces données ont permis de comparer les concentrations en toxines réellement observées dans les tissus (TOX) avec le taux d'accumulation théorique des toxines (TAR) calculé à partir des relations linéaires établies entre OAR, nombre de cellules, fluorescence et toxicités. Les résultats montrent des coefficients de corrélation élevés (R^2 = 0,78) aussi bien pour FTA/TAR que pour FTA/TOX, à la fois pour les huîtres et pour les coquilles Saint-Jacques. La relation TAR/TOX, une fois validée, permettrait d'approcher la notion de quantité minimale de toxine absorbée pour déclencher le processus d'accumulation de toxines.

Keywords: Paralytic shellfish poisoning; Feeding activity; *Crassostrea gigas*; *Alexandrium minutum*

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† This paper is dedicated to Serge Bougrier who died suddenly in late October 2001.

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1. Introduction

Filter-feeding bivalves, depending on the species, do not have the same capacities for accumulation of the paralytic toxins produced by certain dinoflagellates of the genus *Alexandrium*. Thus, oysters take up less toxins than mussels when both are exposed simultaneously to a toxic algal bloom (Shumway et al., 1990; Lassus et al., 1999). Reduced filtration and prolonged valve closure are often regarded as external physiological manifestations of inhibited consumption in bivalves in the presence of paralytic shellfish poison (PSP) producers (Bricelj and Shumway, 1998; Shumway and Cucci, 1987). However, as shell valve activity can also be related to respiration, its relevance as a physiological parameter for controlling the effects of a toxic diet is debatable (Lassus et al., 2000). Moreover, Bricelj and Shumway (1998) indicate that bivalves, despite moderate to poor absorption efficiency, can attain high accumulation efficiencies. On the basis of these observations and those of various authors, Bricelj and Shumway (1998) sought to determine which bivalve species are "sensitive" (slightly contaminated) and which relatively insensitive (highly contaminated). However, even when feeding activity is undisturbed and the ingestion rate high, a low weight ratio between viscera and total meat can be responsible for moderate to low contamination rates for the whole animal. The preferential accumulation of paralytic toxins in some organs (digestive gland, kidneys) could account for this apparent paradox. This is particularly the case for pectinids, as the low correlation between adductor muscle and digestive gland toxicities makes it difficult to predict whether the meat is safe or not for human consumption (Beitler, 1991; Lassus et al., 1992; Cembella et al., 1994).

Previous experimental studies showed a certain sensitivity of the Pacific oyster *Crassostrea gigas* to different toxic diets based on *Alexandrium* spp. (Bardouil et al., 1993; Lassus et al., 1996, 1999), in particular, a slowing down of filtration and feeding rates and of faeces production. Maximal contamination levels of 450–500 µg eq. STX 100 g^{-1} were obtained after 10–15 days of monospecific feeding with *Alexandrium minutum* at a concentration as low as 120 cell ml^{-1} (Lassus et al., 1999). With the same treatment, king scallops (*Pecten maximus*) of commercial size reached a somewhat lower contamination level (350 µg eq. STX 100 g^{-1}), but then displayed much slower detoxification kinetics. In fact, total toxicity in the scallop was still higher than the salubrity threshold (80 µg eq. STX 100 g^{-1}) after 2 weeks, but dropped below this threshold in the oyster at the fourth day of detoxification (Bougrier et al., 2001; Lassus et al., 2002).

These observations suggested reactions specific to each of these shellfish species in the presence of a toxic diet with *A. minutum*, which led us to search for parameters indicative of these specificities.

The recent development of sequential data acquisition techniques in feeding physiology allowed us to perform continual fluorescence measurements in vivo at the outlet of individual units containing an experimental bivalve. Measurement of feeding time activity (FTA) appeared to be a useful parameter for correlating individual feeding activity and toxin accumulation rates (Bougrier et al., 2001).

Moreover, on the basis of relations established between absorbed organic matter, ingested organic particulate matter, the number of corresponding phytoplankton cells and (in the case of toxic plankton) the amount of toxin per cell, it seemed useful to convert values for absorbed organic matter into toxin values and to compare the results obtained with toxicity values measured by chemical analysis. If a simple relation can be established between calculated and measured values, it may eventually be of interest to replace direct quantification of toxins involving a chemical procedure (both onerous and costly) with a simpler indirect method based on measurement of absorbed particular organic matter.

2. Methods

2.1. Biological materials

Oysters (*C. gigas*) and scallops (*P. maximus*) were obtained, respectively, from Bourgneuf Bay producers and Brest Bay fishermen. Neither of these areas is known for blooms of paralytic toxin-producing microalgae. The individuals (in sexual resting phase) had a mean wet weight of 51.3 ± 5.1 g for oysters and 76.2 ± 7.0 g for scallops. Before transfer into experimental units, the animals were acclimated for 5–6 days in natural seawater (16 ± 0.5 °C) containing *Scrippsiella trochoidea*, a non-toxic dinoflagellate of the same size and energy value as *A. minutum* (Bardouil et al., 1993). Cultures of *A. minutum* were grown at 16 ± 1 °C, with a light intensity of 50 ± 4 µmol photons m^{-2} s^{-1} and a 12 h/12 h light/dark photoperiod. The AM89BM strain of *A. minutum* isolated in Morlaix Bay in 1989 had a mean toxicity in culture of 2.4 ± 0.1 pg eq. STX per cell at the end of the exponential growth phase. The cultures were used at this stage, and toxicity was re-evaluated on an aliquot of each new culture flask used during experiments, i.e. every day.

2.2. Experimental device

The individual responses of each shellfish species to a continuous supply of *A. minutum* up to a mean concentration of 120 cell ml^{-1} were assessed using an experimental recirculating flume. This system consisted of six 1-l units (five living oysters or scallops plus an empty shell as control) continuously supplied with seawater pumped from a natural 100 l reserve. The *A. minutum* culture was injected into the seawater reserve via a peristaltic micropump that started or stopped automatically depending on the in vivo fluorescence level in the tank.

Continuous measurement of fluorescence by a Turner 10AU 072 fluorometer was integrated via an acquisition and control card (AD Clone interface) connected to a PC. This

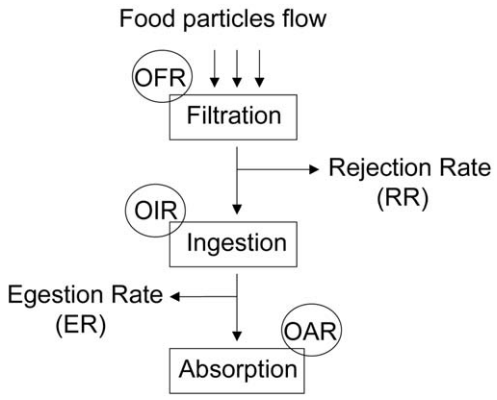

Fig. 1. Conceptual scheme for oyster and scallop feeding process on organic material issue from toxic phytoplankton. OFR, organic filtration rate; OIR, organic ingestion rate; OAR, organic absorption rate; RR, rejection rate (pseudofaeces production); ER, egestion rate (faeces production).

system allowed a constant *A. minutum* concentration (120 cell ml^{-1}) to be maintained throughout the system after calibration by discrete counts using a microscope or Coultronics particle counter.

The *A. minutum* concentration was equivalent to 0.5 mg 1^{-1} of total particulate matter (TPM), i.e. the amount required to obtain a toxin concentration above the salubrity threshold (80 µg eq. STX 100 g^{-1} of meat) at the end of the contamination period (10–15 days).

Residual seawater at the outlet of each unit containing bivalves was returned to the reserve and then recirculated in the circuit. The pump supplying the circuit had a flow capacity of 400 l h^{-1}.

Each experimental unit was connected by an electromagnetic valve to a micropump, so that an aliquot of seawater + *A. minutum* was pumped for 1 min, every 6 min, to a Turner fluorometer for analysis of fluorescence level. The procedure for opening and closing the six electromagnetic valves was regulated by a control/acquisition programme. Thus, fluorescence units were recorded continuously, and measurements of the differences between control fluorescence and that of each individual indicated whether *A. minutum* cell retention by each individual was ≥5%. Thus, FTA was defined as the percentage of time (relative to the entire experimental period) actually used by the animal to filter food particles with a retention of ≥5%. This parameter was independent of the value for the fluorescence difference between the control and experimental animals. The organic absorption rate (OAR) was such that OAR = the organic filtration rate (OFR) minus the organic egestion rate (Fig. 1). OAR and OFR were evaluated by the biodeposits method (Hawkins et al., 1996; Urratia et al., 1996; Iglesias et al., 1992). From OAR, the toxin concentration per cell, and the ratio between POM and cell concentrations, it was possible to calculate the toxin accumulation rate (TAR). The ratio between the toxicity observed by chemical analysis (TOX) and the calculated accumulation rate (TAR) allowed toxin accumulation efficiency (TAE) to be determined: TAE = 100 × (TOX/TAR).

2.3. Chemical analysis

Paralytic phycotoxins were analysed in each individual by ion-pairing high performance liquid chromatography (IP-HPLC) at the end of the contamination period, according to the method of Oshima (1995). Total meat was minced in CH_3COOH 0.1 N at 4 °C on a V/W basis. After centrifugation (3000 × *g*, 15 min, 4 °C) the pH of extracts was adjusted to 3.0–3.5 with glacial acetic acid. After half-dilution, supernatants were ultrafiltered (20 000 Da, Sartorius Centrisart) and stored at 4 °C until analysis. For *A. minutum* cultures, 10 ml were pipetted at the end of the exponential phase. After centrifugation, cell concentrates were suspended in CH_3COOH 0.1 N and frozen at –80 °C. Extraction was performed according to the protocol of Ravn et al. (1995).

Diluted solutions of each compound of PSP1-B reference material (MACSP, NRC-Halifax, Canada) were used as external standards for quantitative detection. In consideration of the dilution factors, the molar concentration was converted into µg STX Eq. 100 g^{-1} of bivalve meat by using the conversion factors of Oshima (1995), i.e. 297 and 168 µg STX eq µM for GTX3 and GTX2, respectively.

Correlation trials were performed using Excel 97 software, and validation of data adjustment by the "Lack of Fit" test using StatGrafic software.

3. Results

During five experiments in individual units (Table 1), 35 oysters (*C. gigas*) and 15 scallops (*P. maximus*) were fed continuously for 9–14 days with *A. minutum* at a concentration of 120 cell ml^{-1}.

Considerable inter-individual variability of FTA (expressed in percentages of time) was noted in oysters and scallops. By way of example, representative recordings for some selected oysters are shown (Fig. 2). Some animals had relatively constant (high or low) FTA values over time (O24), whereas the values for others were quite variable (O23), or decreasing (O11), or increasing (O25). It is noteworthy that

Table 1
Characteristics of each individual experiment monitoring *A. minutum* contamination: bivalves studied and contamination periods

Experimental periods	Length (days)	Animals	Number of animals by experiment	Mean wet weight (g) ± standard deviation
March 1998	9	Scallops	5	14.45 ± 2.10
October 1999	13	Scallops	10	26.12 ± 4.72
March 1999	14	Oysters	10	4.23 ± 1.11
March 2000	11	Oysters	10	3.91 ± 0.91
March 2001	9	Oysters	15	5.60 ± 1.23

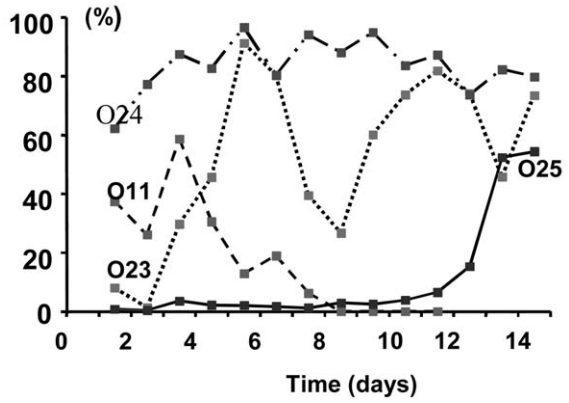

Fig. 2. Examples of FTA changes in four oysters during the contamination phase; (activity as percentage of time, see text).

oyster O25 only began real feeding activity on the 12th day of the experiment.

In terms of the total number of experiments performed, it may be noted that oysters were on average involved in feeding activity only during half of the contamination period (FTA = 47.6 ± 24.7% for n = 20).

The variations of animal wet weight and the different experimental periods used (9–14 days of contamination) required that all results be reported per gram of wet weight and per day. Expressed in these units, the international threshold for the maximal acceptable concentration in an animal was 0.8 µg of toxins per gram of meat wet weight. Thus, an animal was considered as "positive" if it accumulated at least 0.8 µg of toxins, and "negative" otherwise.

For the oyster, when a linear regression is fitted to describe the relationship between either FTA and TAR, or (Fig. 3) FTA and TOX, the R^2 value indicates that the model accounts, respectively, for 78% and 78.2% of the variability. P-values in the ANOVA table indicate statistically significant relationships at the 99% confidence level. Besides, the Lack-of-Fit test (comparison of the variability of current model residuals with that between observations at replicate values of the independent variable X) indicates that the model is adequate for the observed data. Trend curves corresponding to other types of models were also tested, which sometimes gave higher R^2 values, but with similar or less relevant Lack-of-Fit P-values. Therefore, in view of the individual variations noted above and to facilitate inter-species comparisons, a linear type relation was used for subsequent investigations:

$$\mathrm{TAR} = \mathrm{a\ FTA} + \mathrm{b} \qquad (1)$$

It seemed of interest to detect a possible relation between daily TAR and daily rates of toxin accumulation (TOX) in order to evaluate the predictive capacities of such a relationship.

The linear model for oysters (Fig. 4) shows that TAR accounts for 73.9% of TOX variability, with a statistically significant relationship at the 99% confidence level. However, the Lack-of-Fit test indicates no adjustment at the 95% level. When tested, other curvilinear models gave a higher R^2 value (e.g. the double reciprocal model), but without subse-

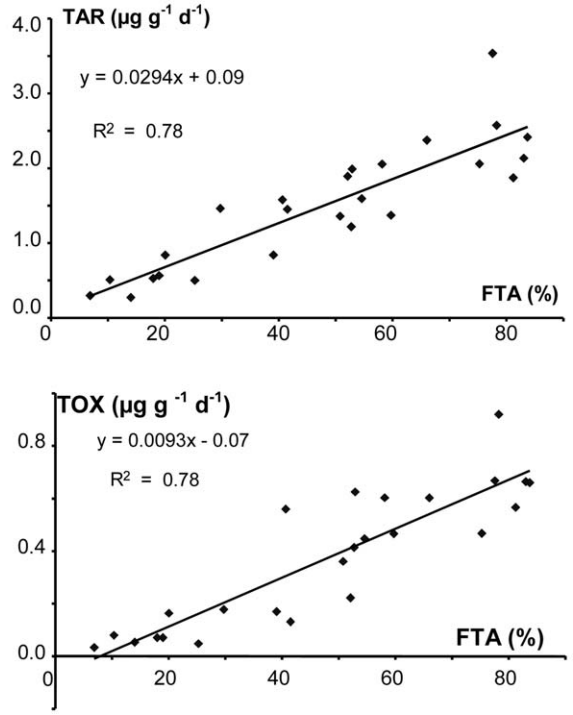

Fig. 3. Relations between FTA and i) the calculated daily amount of toxins accumulated (TAR), and ii) the observed daily amount of toxins actually accumulated by oysters (TOX).

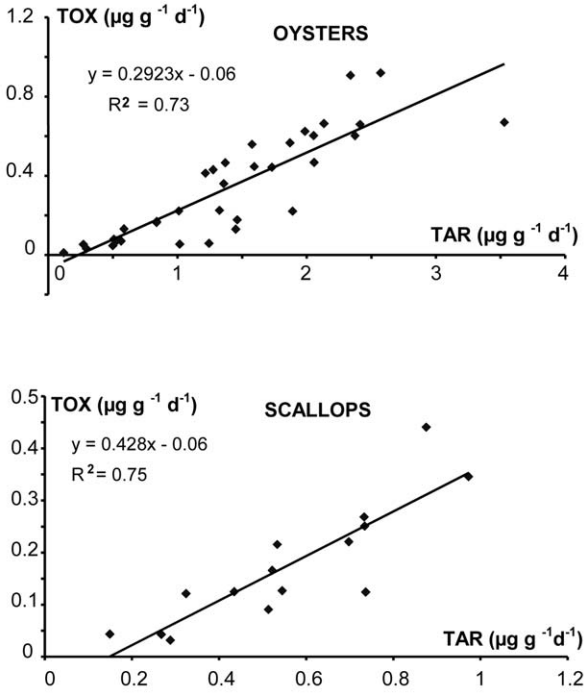

Fig. 4. Relations between calculated (TAR) and observed (TOX) daily amounts of toxin accumulated, respectively, in oysters (above) and scallops (below).

quent change in Lack-of-Fit *P*-values. For scallops (Fig. 4), on the other hand, the R^2 value indicates that the model, as fitted, accounts for 75.2% of TOX variability.

As the *P*-value for Lack-of-Fit in the ANOVA table (0.17) is greater than 0.10, the king scallop model appears to be adequate for the observed data.

Although other types of models accounted better for the relation between TAR and TOX, the linear model was actually adopted:

$$TOX = a' \ TAR + b' \quad (2)$$

This relation seems adequate for scallops and accounts for at least 73.9% of TOX variability in oysters. After further validation, it should allow the determination of two specific values: the TAR value when TOX = 0 and the regression curve slope.

4. Discussion

The variations of sensitivity, and thus of the accumulation potential of different bivalve species for paralytic toxins, have been known for some years. They were first quantified by bioassay on isolated nerve (Twarog et al., 1972), Other studies subsequently showed a more or less marked correlation with valve activity (Lassus et al., 2000), but especially a close relation with ingestion and absorption rates (Bricelj et al., 1990, 1991; Wisessang et al., 1991; Bricelj and Shumway, 1998; Bougrier et al., 2001). This relation is easier to establish experimentally, to the extent that the diet is composed exclusively of toxic microalgae, thereby simulating in a certain manner the conditions referred to as “pure culture”, corresponding to a “red tide” situation in the natural environment.

Quantitative IP-HPLC, analyses of toxin concentrations in bivalves are relatively complicated. Although they are suitable for assessing the amount of toxin taken up at the end of an experiment, as well as the toxin profile, it is difficult to estimate toxicity over time (accumulation kinetics), unless the study involves large populations. Thus, an indirect estimation of toxicity, particularly the rate of toxin accumulation in tissues, could be useful when the cellular toxicity of the dominant phytoplankton species is known. A linear slope relation of (1) between observed and calculated toxicity should allow this type of approach to be developed. In fact, a readjustment of model (2) relative to TAR values allows a linear correlation to be obtained between observed and calculated values (with a slope close to 1). This facilitates evaluation of theoretical toxicities from ecophysiological monitoring of the animal (biodeposits method) and provides a better understanding of cell toxicity in experimental conditions in cultures and/or during observations in situ.

Moreover, the direct relation between TAR and TOX, provided that it is linear, shows that the regression curve does not pass through the origin and that the TAR value corresponding to the intersection of the curve with the *X* axis can be considered as indicative of the amount of absorbed toxins needed to induce accumulation. In other words, this minimal amount serves as a substrate for the basal metabolism of the animal, while the excess amount is taken up for subsequent use in production processes.

Further studies are required to refine this approach, particularly to confirm the exact nature of the TAR/TOX relation.

The proportion of toxins accumulated in tissues relative to the amount theoretically absorbed (TAE) was estimated in our experimental conditions to be 20% and 30%, respectively, for the Pacific oyster and the king scallop. Thus, these values are lower than the TAE calculated for the blue mussel (75–79%, Bricelj et al., 1990) and the green mussel (50%, Wisessang et al., 1991), but close to that for the northern quahog *Mercenaria mercenaria* (35–40%, Bricelj et al., 1991).

These initial results would suggest different metabolic pathways for PSP toxins accumulation/elimination by bivalves. Specifying the minimal amount of absorbed toxin required to induce accumulation process could be worth being explored when comparing bivalve specific responses to PSP toxins contamination.

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